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Article

The Sphingosine Kinase-Sphingosine-1-Phosphate Axis Is a Determinant of Mast Cell Function and Anaphylaxis

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SUMMARY

Sphingosine-1-phosphate, a key mediator in immune cell trafficking, is elevated in the lungs of asthmatic patients and regulates pulmonary epithelium permeability. Stimulation of mast cells by allergens induces two mammalian sphingosine kinases (Sphk1 and Sphk2) to produce sphingosine-1-phosphate (S1P). Little is known about the individual role of these kinases in regulating immune cell function. Here we show that in mast cells, Sphk2 is required for production of S1P, for calcium influx, for activation of protein kinase C, and for cytokine production and degranulation. However, susceptibility to in vivo anaphylaxis is determined both by S1P within the mast cell compartment and by circulating S1P generated by Sphk1 predominantly from a non-mast cell source(s). Thus, sphingosine kinases are determinants of mast cell responsiveness, demonstrating a previously unrecognized relationship with anaphylaxis.

INTRODUCTION

Sphingolipids are necessary constituents of membranes found to concentrate in liquid ordered domains (commonly referred to as lipid rafts or cholesterol-enriched membrane microdomains). Multiple cell-membrane receptors control the activity of sphingosine kinases (SphKs), which generate the lysosphingolipid sphingosine-1-phosphate (S1P) and modulate the amounts of the sphingosine (SPH) and ceramide (Spiegel and Milstien, 2003). These sphingolipid metabolites are bioactive lipids that can directly bind proteins, activate signaling pathways, and affect cellular responses (Hannun et al., 2001; Olivera and Spiegel, 2001).

S1P is an intracellular mediator of cell function and is also an extracellular ligand for a family of five G protein-coupled receptors, named S1P receptors ($S1P_{1-5}$) (Sanchez and Hla, 2004). Recent studies that used the immunosuppressive drug FTY720, a structural analog of SPH that is phosphorylated in vivo by SphK creating a S1P mimetic, revealed an essential role for S1P and its receptors in lymphocyte trafficking (Rosen and Goetzl, 2005). Exit of thymocytes from the thymus is controlled by upregulation in $S1P_1$ expression during the maturation of thymocytes, which mediates the chemotactic response to S1P (Matloubian et al., 2004; Schwab et al., 2005). Drugs interfering with the function of the S1P-S1P receptor axis show promise in transplantation and in clinical trials involving patients with relapsing multiple sclerosis (Massberg and von Andrian, 2006). Furthermore, alteration of the S1P gradient by dietary components impacts on lymphocyte egress and immunity (Schwab et al., 2005), highlighting the importance of preserving in vivo S1P homeostasis. In certain inflammatory conditions such as asthma and rheumathoid arthritis, local increases in the amounts of S1P in the airways (Ammit et al., 2001) and in the arthritic joints (Kitano et al., 2006), respectively, have been reported. S1P receptors are ubiquitously expressed in a variety of cell types, and S1P dramatically alters the function of endothelial, epithelial, and smooth muscle cells (Ammit et al., 2001; Gon et al., 2005; Olivera and Spiegel, 2001; Spiegel and Milstien, 2003), so S1P may participate in the pathophysiology of these inflammatory diseases.

Mast cells are potent producers of intracellular and secreted S1P via SphK activation. Mast cells express $S1P_1$ and $S1P_2$ receptors on their cell surface (Olivera and Rivera, 2005). Similar to T cells, $S1P_1$ is important for mast cell chemotaxis. In contrast, $S1P_2$ contributes to the robustness of the mast cell degranulation response (Jolly et al., 2004). S1P has also been suggested as an important regulator of calcium mobilization in mast cells, through an unknown mechanism that is seemingly independent of S1P receptors (Choi et al., 1996; Lee et al., 2005a; Melendez and Khaw, 2002). The balance of SPH to S1P has also been implicated as a rheostat that controls the mast cells responsiveness to a stimulus (Prieschl et al., 1999). This view predicts that SphK activation is a key checkpoint in mast cell function and that it should be closely linked to cell-surface receptor stimulation. This was demonstrated

to be the case for the high-affinity receptor for IgE (FcεRI): we previously found that activation of both SphK1 and 2 was dependent on FcεRI-mediated activation of Fyn and Lyn (Olivera et al., 2006; Urtz et al., 2004), the Src family kinases that are promptly activated upon receptor engagement (Rivera and Gilfillan, 2006). However, the precise role of each SphK isoform in mast cells has not been conclusively demonstrated. Furthermore, because S1P is present in plasma in relatively high amounts and extracellular S1P in vitro can influence mast cell responses, it was important to understand whether the contributions of these kinases to mast cell function can be compensated in vivo by S1P in the surrounding environment.

By using mice in which SphK1 and 2 genes were individually or jointly deleted, we investigated their role in mast cell function. Several unanticipated findings resulted from this analysis. SphK2, and not SphK1, was found to be the main regulator of S1P production, mediating calcium influx, NF-κB activation, cytokine production, and degranulation of mast cells. In contrast, SphK1 deficiency did not markedly alter mast cell signals or function; however, mice deficient in this gene had greatly reduced amounts of circulating S1P. In vivo anaphylaxis revealed that circulating amounts of S1P were important in determining mast cell responsiveness and demonstrated a partnership between SphK1 and SphK2 as extrinsic and intrinsic regulators, respectively, of mast cell responsiveness. This previously unrecognized in vivo role for SphK-generated S1P may be a key determinant of anaphylaxis.

RESULTS

Characterization of SphK-Deficient Mast Cells

To assess the role of the individual sphingosine kinases (SphK1 and SphK2) in mast cells, we utilized genetically altered mice in which the individual genes were deleted or both genes (*Sphk1* and *Sphk2*) were jointly deleted. The deletion of both genes results in embryonic lethality (Mizugishi et al., 2005), so we harvested mast cell progenitors from the liver of 11.5-day-old embryos for each genotype and differentiated these cells in culture with IL-3 and stem cell factor (SCF) (Kovarova et al., 2006; Saitoh et al., 2000). Alternatively, in vivo studies on *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice as well as in vitro analysis of bone marrow-derived mast cells (BMMCs) from these mice were also conducted (see following sections). The differentiated cells from each genotype showed equivalent cell-surface FcεRI expression and absence of mRNA for the corresponding targeted gene (Figures 1A and 1B). By using an assay that distinguishes the activity of the two kinases (Liu et al., 2000; Olivera et al., 2006), we found the appropriate kinase activity to be reduced (Figure 1C). As previously described (Olivera et al., 2006), some overlap (~25%) of SphK activity was seen in this assay. This overlap in activity likely explains the higher-than-expected SphK1 activity observed in *Sphk1*$^{-/-}$ mast cells, because SphK2 activity was approximately 2-fold greater. Measurement of sphingosine (SPH) and sphingosine-1-phosphate (S1P) amounts indicated that the absence of SphK2, but not SphK1, resulted in increased amounts of SPH and decreased S1P production, demonstrating that this was the dominant kinase producing S1P in these cells (Figures 1D and 1E).

Impaired Mast Cell Effector Functions in SphK2-Deficient Cells

The role of SphK1 or 2 on mast cell responses was explored. The loss of SphK2, but not SphK1, caused a marked inhibition (>60%) in the FcεRI-dependent degranulation (Figure 2A). This inhibition in *Sphk2*$^{-/-}$ cells could be partly restored by concomitant addition of S1P as a stimulus (see Figure S1 in the Supplemental Data available online). Deficiency of both SphKs did not augment this inhibition (Figure 2A). Measurement of TNF, IL-6, and IL-13 secretion also revealed a dependence on SphK2 (Figures 2B–2D). Importantly, normal cytokine secretion was restored when *Sphk2*$^{-/-}$ or *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells were treated with the generic stimulus of phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187. As shown in Figures 2E and 2F, both arachidonic acid (AA) release and leukotriene B_4 (LTB_4) production were impaired in *Sphk2*$^{-/-}$ mast cells. These findings demonstrate a role for SphK2 downstream of FcεRI and distinguish the role of this kinase from that of SphK1 in regulating mast cell effector responses.

SphK2-Deficient Mast Cells Have Defective Calcium Influx

The impaired degranulation, cytokine secretion, and arachidonic acid release suggested that SphK2 activity might impact on a signaling element in common to these three responses. SphK1 and S1P have been implicated in mast cell calcium mobilization from intracellular stores (Choi et al., 1996; Melendez and Khaw, 2002). Considerable evidence argues for a dominant role of inositol (3,4,5)-trisphosphate (IP_3), rather than S1P, in mobilizing calcium in mast cells, as well as other immune cells (Michell et al., 1992). Conversely, other studies suggested that the FcεRI principally utilized SphK to mobilize calcium (Choi et al., 1996; Melendez and Khaw, 2002).

Analysis of the FcεRI-dependent calcium response revealed an impairment in the extent of the response in the absence of SphK2, but not SphK1 (Figure 3A). Cells in which both kinases were absent showed no further reduction, demonstrating a dominant role for SphK2 in regulating calcium. This was further supported by experiments in which WT or *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells were treated with N,N-Dimethylsphingosine (DMS), a competitive inhibitor of SphK activity (Edsall et al., 1998; Liu et al., 2000). DMS similarly reduced the calcium response in WT cells, but had minor effects in *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells (Figure S2). We explored whether the SphK2 effect was through IP_3 production, which causes calcium release from endoplasmic reticular stores. SphK deficiency, in general, had no significant effect on mast cell IP_3 production (Figure 3B). Tyrosine phosphorylation of PLCγ1 and PLCγ2, the enzymes that produce IP_3 in mast cells (Saitoh et al., 2003), was normal, though some minimal

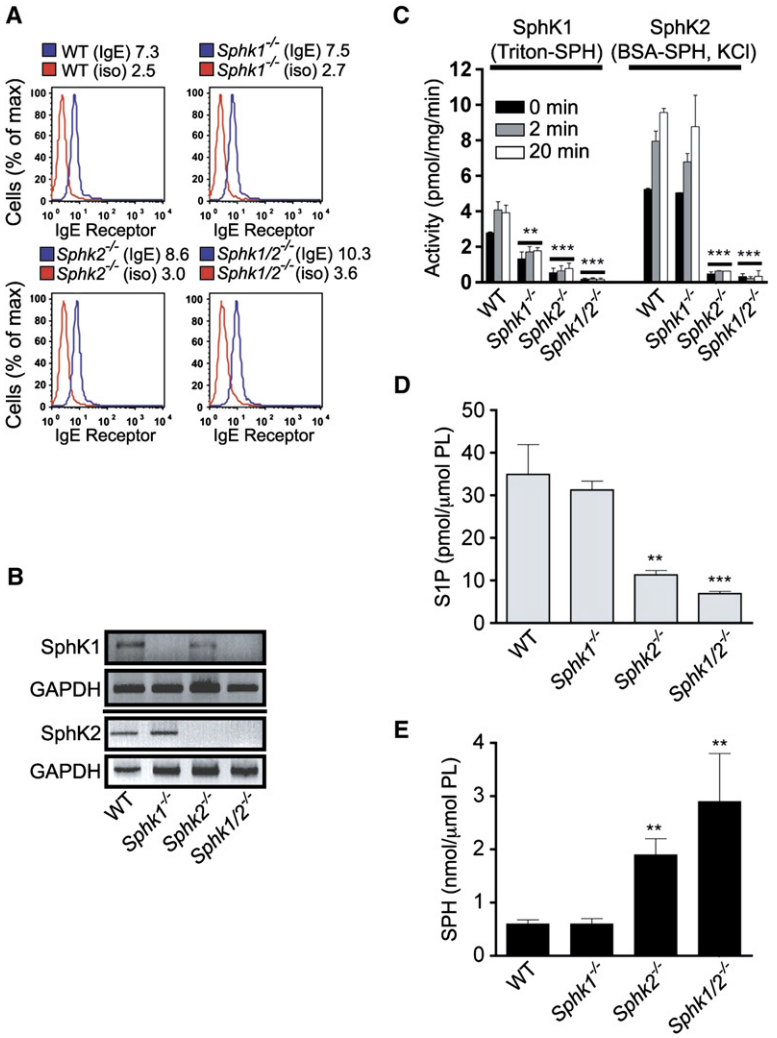

Figure 1. Characterization of Sphingosine Kinase-Deficient Fetal Liver-Derived Mast Cells

(A) Cell-surface expression of FcεRI in *Sphk1*$^{-/-}$, *Sphk2*$^{-/-}$, and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells as compared to WT mast cells. Cells were incubated with FITC-labeled IgE and fluorescence intensity was measured by flow cytometry. The red line represents the isotype control that used FITC-rat IgG1 instead of FITC-IgE. The numbers indicate the mean fluorescence intensity of each sample.

(B) RT-PCR analysis of expression of SphKs mRNA.

(C) SphK1 or 2 activities from cells were determined by means of assay conditions that favor SphK1 (in the presence of Triton-micelles) or SphK2 (in the presence of higher salt concentrations) as described in Experimental Procedures.

(D and E) Cellular S1P (D) and SPH (E) were measured as described (Olivera et al., 2006). Data represent the mean ± SEM of 3 to 4 independent experiments. Statistical significance relative to WT cells was $^{**}p < 0.01$; $^{***}p < 0.001$.

loss of phosphorylation was observed for PLCγ2 when both SphKs were absent (Figure S2).

Because IP_3 production appeared to be normal, we explored whether the loss of SphK2 was impinging on intracellular calcium mobilization independently of IP_3. By removing calcium from the extracellular medium, the FcεRI-dependent release of Ca^{2+} from intracellular stores can be measured and was found to be normal among the different genotypes (Figure 3C). In contrast, replenishment of Ca^{2+} in the medium, after 10 min of FcεRI stimulation in the absence of Ca^{2+}, revealed a defect in its uptake for *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells, but not for *Sphk1*$^{-/-}$ cells. This defect in calcium influx was further confirmed by direct measurement of $^{45}Ca^{2+}$ uptake, which demonstrated a marked (>60%) inhibition for *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells (Figure 3D). However, an artificial increase of intracellular calcium by ionophore A23187, in combination with PMA, was able to fully restore degranulation in *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells (Figure 3E), demonstrating that defective degranulation could be restored by increasing the intracellular calcium concentration and activating PKC.

SphK2-Deficient Mast Cells Show Defective PKC Translocation and NF-κB Activation

Calcium is necessary for the activation of the calcium-binding isoforms of PKC, of which the membrane translocation and function of PKCβ has been demonstrated as important for mast cell degranulation and IL-6 production (Nechushtan et al., 2000; Ozawa et al., 1993). Thus, we explored whether loss of SphK2 affected the translocation of phosphorylated PKCs in mast cells. The translocation of membrane-localized S660-phosphorylated PKCs α and β (which reflects mature and active PKC [Behn-Krappa and Newton, 1999]) was impaired in *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells. Membrane translocation and phosphorylation of these PKC isoforms was not defective in *Sphk1*$^{-/-}$ mast cells. These results demonstrate that SphK2 is essential for normal targeting of active PKCα and β to mast cell membranes where they may exert their exocytotic function (Ozawa et al., 1993).

Because cytokine production was impaired by SphK2 deficiency, and TNF and IL-6 production is NF-κB dependent in mast cells (Marquardt and Walker, 2000), we explored the role of SphKs in NF-κB activation. We observed

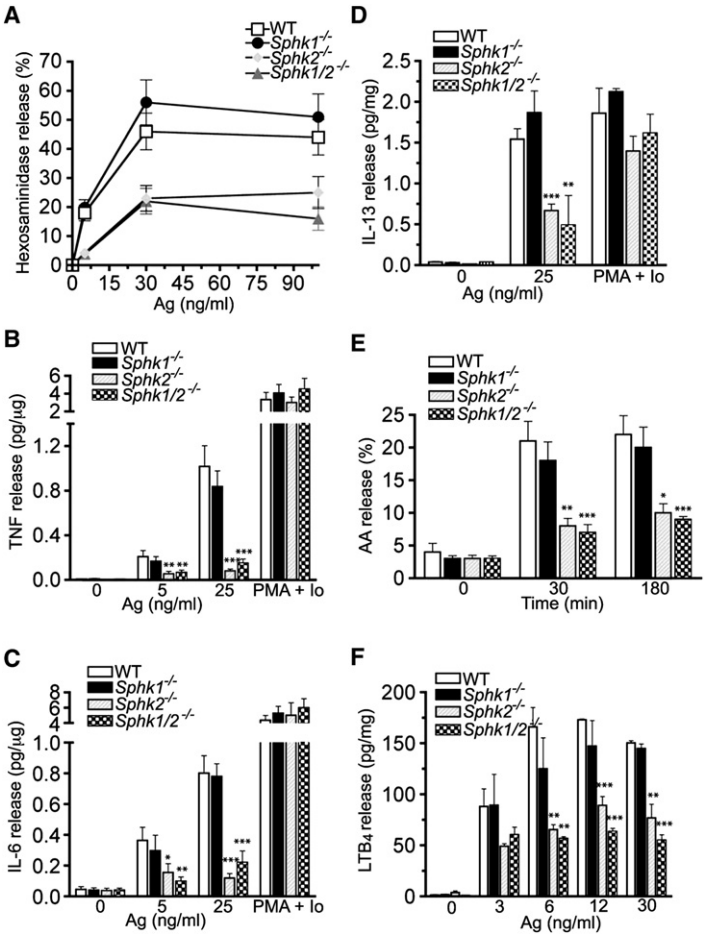

Figure 2. SphK2-Deficient Fetal Liver-Derived Mast Cells Have Defective Effector Responses

(A) Cells of the indicated genotype were stimulated via FcεRI, and degranulation was measured by release of the granule enzyme β-hexosaminidase. The fraction of enzyme released in the medium after stimulation is expressed as a percent of total cellular content. (B–D, F) Cytokine (B–D) or leukotriene (F) B_4 release into the extracellular media was quantitatively measured by specific immunoassays. (E) Mast cells were loaded with ^{14}C-labeled arachidonic acid (AA) before FcεRI stimulation. The released AA detected in the extracellular medium is expressed as the percent of total ^{14}C-labeled AA incorporated in the cell. Data are the mean ± SEM of 3 to 5 independent experiments. Statistical significance relative to WT cells was *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

a marked defect (>60%) in nuclear NF-κB activity in *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mast cells, but not in *Sphk1*$^{-/-}$ mast cells (Figure 4B). Normal NF-κB activity was restored when *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ cells were treated with PMA and calcium ionophore A23182 (Figure 4C), demonstrating a calcium- and PKC-mediated restoration. Assembly of the CARMA1-Bcl10-Malt complex required for NF-κB activation in T and B cells is dependent on PKCθ and β, respectively (Lee et al., 2005b; Wang et al., 2004). In mast cells, which PKC isoform is responsible for NF-κB activation is unknown (Klemm et al., 2006; Rivera, 2006a), although PKCβ is a likely candidate (Nechushtan et al., 2000).

The activation of MAP kinases, which are known to regulate cytokine expression, was also studied. These kinases were normally activated (Figure 4D), demonstrating that SphKs are not essential for their activity.

Regulation of Circulating S1P by SphK1 and Intrinsic Mast Cell SphK2 Activity Determine the Anaphylactic Response

To test the in vivo role of SphKs, we conducted a passive systemic anaphylactic challenge of mice (Saitoh et al., 2000). Challenge of WT mice resulted in histamine release ranging from 10 to 30 μM, and analysis of the serum concentrations of S1P in these mice revealed a strong association between the concentration of this sphingolipid and the concentration of circulating histamine (Figures 5A and 5B). In vivo challenge of *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice revealed a defect in the anaphylactic response in *Sphk1*$^{-/-}$ but not *Sphk2*$^{-/-}$ mice (Figure 5B). The latter mice had increased mast cell numbers, although mast cells from these mice produced little S1P and were less responsive (Figures 1D and 2 and Figures S3 and S4). However, as previously reported (Mizugishi et al., 2005; Zemann et al., 2006) and confirmed herein, *Sphk2*$^{-/-}$ mice had abnormally high amounts of circulating S1P relative to WT mice (>150%, Figure 5C). Given the association of S1P with the concentrations of histamine shown in Figure 5A, and the finding that S1P can influence mast cell responsiveness in vitro (Figure S1; Jolly et al., 2004; Prieschl et al., 1999), the results suggested an extrinsic role of S1P, through SphK1, in regulating mast cell responsiveness in vivo. Supporting this concept, the anaphylaxis-resistant *Sphk1*$^{-/-}$ mice had low concentrations of circulating S1P relative to their WT counterparts (Figure 5C; Allende et al., 2004; Zemann et al., 2006), whereas degranulation of mast cells derived from these mice was completely normal (Figure 2 and Figure S4). To further test this hypothesis, we generated *Sphk2*$^{-/-}$

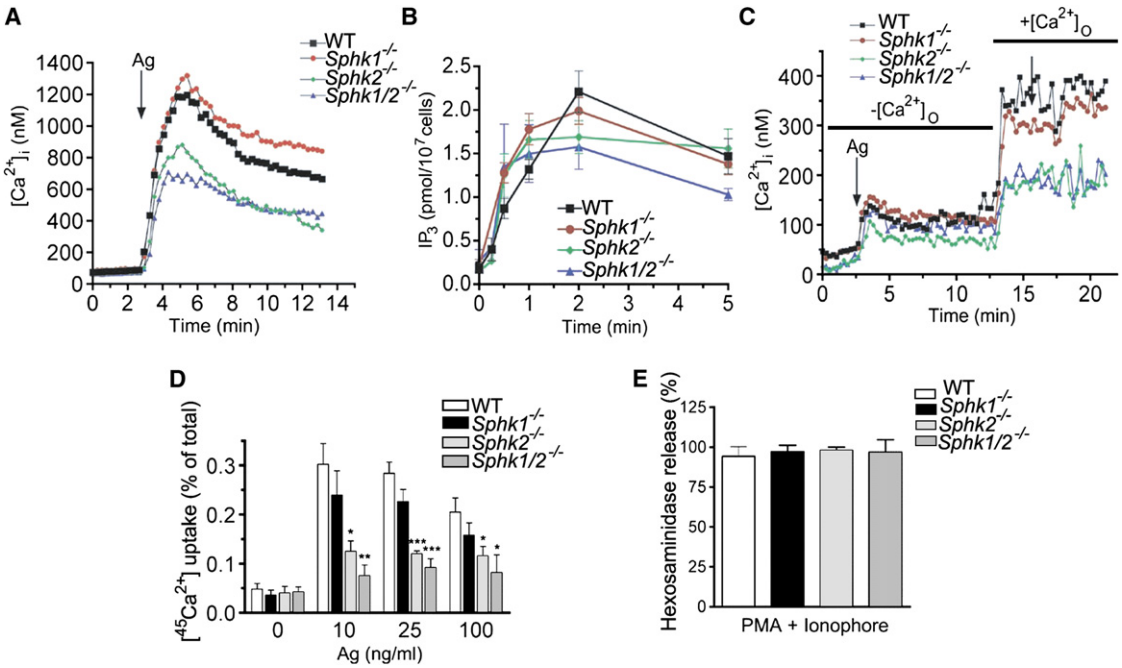

Figure 3. SphK2 Deficiency in Fetal Liver-Derived Mast Cells Reduces FcεRI-Dependent Ca^{2+} Influx but Not the Generation of IP3 and Intracellular Calcium Mobilization

(A) Mast cells with the indicated genotype loaded with the FURA-2AM were stimulated via FcεRI. FURA-2AM flouresence at 510 nm was measured by rapid excitation between 340 and 380 nm. Maximum and minimum emissions were used to calculate the intracellular calcium concentrations.

(B) IP_3 production was measured after FcεRI stimulation. Intracellular IP_3 was extracted and quantified as described in Experimental Procedures. Data are the mean ± SEM of 5 independent experiments (differences were not statistically significant).

(C) FURA-2AM-loaded mast cells were stimulated via FcεRI in the absence of extracellular calcium and flouresence emission was monitored. 10 min after stimulation, 1 mM calcium was added to the medium.

(D) FcεRI-dependent calcium influx was measured by adding $^{45}Ca^{2+}$ to the medium at the time of stimulation. After 10 min, intracellular $^{45}Ca^{2+}$ was determined by scintillation counting and expressed as percent of the total $^{45}Ca^{2+}$ added.

(E) PMA and calcium ionophore A23187 treatment restores the defective degranulation response. Mast cells were stimulated with these agents for 10 min and β-hexosaminidase release to the extracellular medium was determined. Data shown (B, D, E) are mean ± SD of at least three independent experiments. Statistical significance with respect to WT cells was *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mice with one active SphK1 (*Sphk1*$^{+/-}$) allele (*Sphk1*$^{+/-}$ *Sphk2*$^{-/-}$) and *Sphk1*$^{-/-}$ mice with one active SphK2 (*Sphk2*$^{+/-}$) allele (*Sphk1*$^{-/-}$*Sphk2*$^{+/-}$). *Sphk1*$^{+/-}$*Sphk2*$^{-/-}$ mice, unlike their *Sphk2*$^{-/-}$ counterparts, had normal amounts of circulating S1P (Figure 5C), and their mast cells had similarly low SphK activity as those derived from the *Sphk2*$^{-/-}$ mice (Figure S5). However, these mice were now resistant to anaphylaxis (Figure 5B), despite having higher numbers of mast cells (like the *Sphk2*$^{-/-}$ mice) in their tissues (Figure S3). *Sphk1*$^{-/-}$*Sphk2*$^{+/-}$ mice had low amounts of circulating S1P similar to *Sphk1*$^{-/-}$ mice but had mast cells with diminished SphK activity compared to those derived from the *Sphk1*$^{-/-}$ mice (Figure S5). *Sphk1*$^{-/-}$*Sphk2*$^{+/-}$ mice were more resistant to anaphylaxis than *Sphk1*$^{-/-}$ mice (Figures 5B and 5C). Measurement of the SphK activity in the blood for all genotypes demonstrated SphK1 dominance in the circulatory system. As expected, the corresponding reduction of SphK1 activity was observed when this gene was not expressed or when a single allele was active (Figure S5). Moreover, the presence or absence of mast cells in an unchallenged mast cell-deficient mouse (*W*$^{-sh}$/*W*$^{-sh}$) did not alter the amounts of circulating S1P (Figure S6). The in vivo experiments support the view that both SphK1 and 2 play essential roles as extrinsic and intrinsic regulators, respectively, of allergic responses.

DISCUSSION

The findings of this study reveal a key role for SphKs and S1P in mast cell responses and anaphylaxis. Although multiple studies suggested that sphingolipid metabolites might contribute in mast cell activation, direct evidence of a role for such metabolites in mast cell signaling and in vivo function was lacking. Clues for a role in mast cell effector functions were provided by studies reporting increased S1P amounts in the bronchial alveolar lavage of challenged asthmatics (Ammit et al., 2001) and in vitro experiments demonstrating that S1P is required for sustained mast cell degranulation through the $S1P_2$ receptor (Jolly et al., 2004). Thus, understanding the individual roles of SphK1 and SphK2 in mast cell responsiveness and their importance for mast cell function in vivo was necessary.

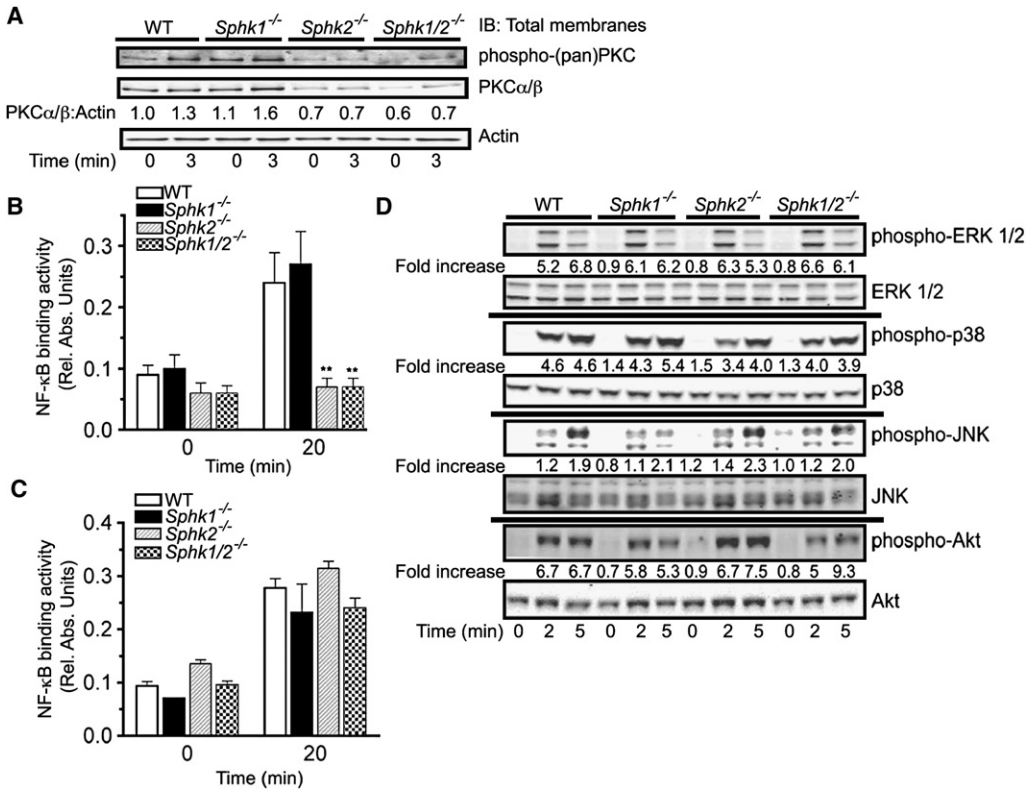

Figure 4. SphK2 Deficiency in Fetal Liver-Derived Mast Cells Results in Defective PKC and NF-κB Activation

(A) FcεRI-stimulated cells were lysed by freeze-thawing and total membranes were isolated. Membrane-translocated PKC (α and β isoforms) and total PKC phosphorylated on S660 was detected by immunoblot with an antibody that recognizes both isoforms and a phospho-pan PKC antibody, respectively. Immunoprobe of the same lysate for actin showed equal protein loading.

(B) Nuclear NF-κB p65 DNA binding activity was measured 20 min after FcεRI stimulation.

(C) Nuclear NF-κB p65 DNA binding activity was also measured from PMA and calcium ionophore A23187-treated cells. Activity is reported as the absorbance at 450 nm. Data are the mean ± SEM from 3 to 5 individual experiments. Statistical significance with respect to WT cells was $^{**}p < 0.01$.

(D) SphK deficiency does not affect MAPK or PI3K-AKT pathways. Phosphorylated proteins in the lysates of FcεRI-stimulated cells were analyzed by immunoblotting with phospho-specific ERK1/2 (Thr202 Tyr204), p38 (Thr180 Tyr182), JNK1/2 (Thr183 Tyr185), and Akt (Ser473), and the respective antibodies recognizing total protein.

This analysis has provided several original and unforeseen findings. SphK2 was found to be an intrinsic regulator of mast cell calcium influx and responses, whereas SphK1 appears to be important in regulating the levels of circulating S1P and thus extrinsically affecting mast cell responsiveness. This is conflicting with the previous suggestions that SphK1 is the key isoform in mast cells (in experiments that used pharmacological inhibitors or siRNA silencing strategies), because its activity has been implicated in calcium responses in the RBL-2H3 mast cell line and in human mast cells (Lee et al., 2005a; Melendez and Khaw, 2002). However, our view is supported by several lines of evidence. (1) Direct measurement of SphK1 and 2 activity in fetal liver-derived mast cells as well as in bone marrow-derived cultured mast cells (BMMC) demonstrated the higher activity of the latter isoform. (2) Both fetal liver-derived mast cells and BMMC from *Sphk2*$^{-/-}$ mice demonstrated defective S1P production and FcεRI-induced degranulation, unlike those of *Sphk1*$^{-/-}$ mice. (3) Fetal liver-derived mast cells from *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ mice mirrored *Sphk2*$^{-/-}$ cells, but not *Sphk1*$^{-/-}$ cells, in both signaling and functional responses. (4) *Sphk1*$^{+/-}$*Sphk2*$^{-/-}$ mice had normal levels of circulating S1P, unlike the *Sphk2*$^{-/-}$ mice, but were resistant to anaphylaxis (in vivo mast cell response). Taken together with the finding of increased mast cell numbers in both *Sphk2*$^{-/-}$ as well as in *Sphk1*$^{+/-}$ *Sphk2*$^{-/-}$ mice, and of approximately equal SphK1 activity in mast cells from these mice, one is led to conclude that the likely determinant for the normal anaphylactic response of the *Sphk2*$^{-/-}$ mouse is the high levels of circulating S1P. This view is also supported by the partial restoration of degranulation for *Sphk2*$^{-/-}$ mast cells when concomitantly stimulated with Ag and S1P. On the other hand, this short-term treatment with exogenous S1P alone (or in combination with Ag) failed to restore the normal rise in intracellular calcium and the defective calcium influx observed in *Sphk2*$^{-/-}$ cells, demonstrating that SphK2 has a role in mast cell functions that are independent of S1P receptors. Altogether, the data implicate SphK2 as a determinant of

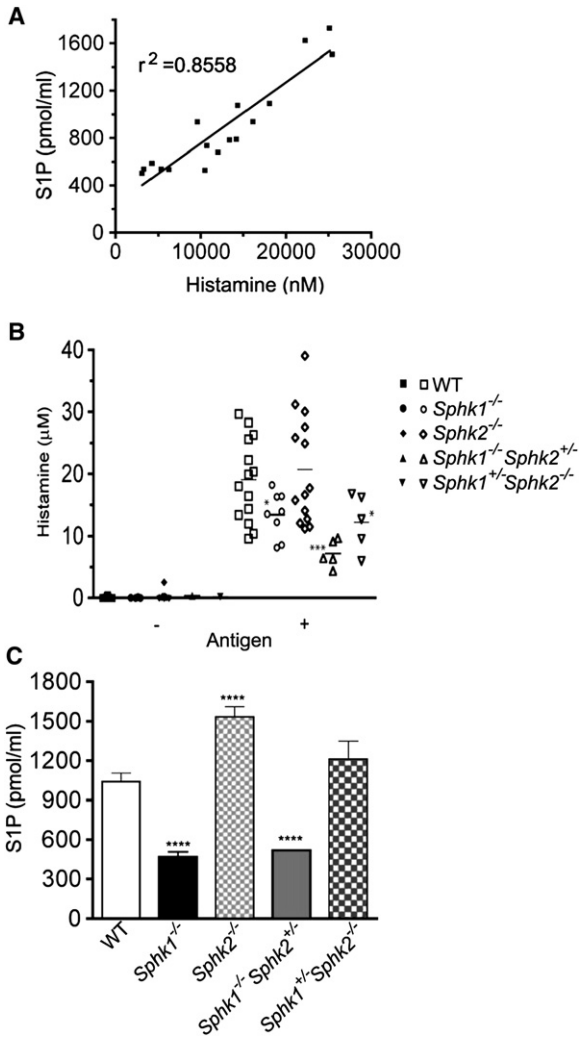

Figure 5. The In Vivo Passive Systemic Anaphylactic Response Is Associated with Circulating S1P Concentrations and Mast Cell SphK2

(A) Correlation between S1P concentrations in serum and plasma histamine concentrations in WT mice after the anaphylactic response. Linear correlation coefficient is shown.

(B) WT, *Sphk1*$^{-/-}$, *Sphk2*$^{-/-}$, and mice deficient for SphK2 and hemizygous for SphK1genes (*Sphk1*$^{+/-}$ *Sphk2*$^{-/-}$) or vice versa (*Sphk1*$^{-/-}$ *Sphk2*$^{+/-}$) were passively sensitized with DNP-specific IgE and challenged 24 hr later with Ag (DNP-HSA) or pseudochallenged with an equal volume of saline. Plasma histamine concentration was measured by competitive ELISA. Statistical significance relative to WT is *$p < 0.04$, ***$p < 0.001$.

(C) S1P in the serum of unchallenged WT, *Sphk1*$^{-/-}$, *Sphk2*$^{-/-}$, *Sphk1*$^{-/-}$ *Sphk2*$^{+/-}$, and *Sphk1*$^{+/-}$ *Sphk2*$^{-/-}$ mice was measured as in a previously described enzymatic assay (Edsall et al., 2000). Statistical significance relative to WT mice was ***$p < 0.001$.

intrinsic mast cell function, whereas SphK1 plays an extrinsic role. Nonetheless, given the diversity and plasticity of mast cells (Galli et al., 2005) in the in vivo environment and the important differences in the gene expression profiles of human and mouse mast cells (Welle, 1997; Saito, 2005), we cannot formally exclude an intrinsic role for SphK1 in mast cells phenotypically different from those used in this study.

Our previous findings (Olivera et al., 2006; Urtz et al., 2004) demonstrated a FcεRI-proximal activation and association of SphKs suggestive of their key role in determining mast cell functional responses. The Src family kinases, Lyn and Fyn, were found to be required for normal activation of SphK activity in mast cells (Olivera et al., 2006; Urtz et al., 2004). Lyn was important for the early onset of SphK1 activity, whereas Fyn was obligatory for the activation of both SphK1 and 2. Interestingly, *fyn*$^{-/-}$ mast cells have a similar phenotype to *Sphk2*$^{-/-}$ mast cells. Both are defective in degranulation and cytokine responses and are unable to activate NF-κB (Gomez et al., 2005; Parravicini et al., 2002; and this study). Ag-stimulated *fyn*$^{-/-}$ mast cells also have markedly decreased S1P amounts relative to WT cells (Olivera et al., 2006), whereas *lyn*$^{-/-}$ mast cells achieve normal S1P levels, though the onset is delayed (unpublished observation). Because *Sphk2*$^{-/-}$ mast cells showed defective calcium influx, we recently revisited this issue in *fyn*$^{-/-}$ BMMCs. These new findings demonstrate that Fyn deficiency also results in decreased calcium influx (J.R., data not shown). Thus, SphK2 is a candidate kinase for regulation of calcium influx downstream of Fyn.

While the detailed mechanism by which SphK2 regulates calcium mobilization remains to be fully explored, it should be noted that unlike a previous suggestion (Lee et al., 2005a), we uncovered no evidence of a role for SphKs or S1P in mobilizing calcium from ER stores. Intracellular S1P formation has been reported to promote calcium-induced calcium entry in human neutrophils (Itagaki and Hauser, 2003) and calcium influx in yeast (Birchwood et al., 2001). In contrast, SPH has been described to inhibit calcium influx (Blom et al., 2005; Condrescu et al., 2002; Mathes et al., 1998; Titievsky et al., 1998). Of particular interest, the study by Mathes et al. (1998) in the tumor mast cell line RBL-2H3 implicates SPH (and its structural analogs) as specific inhibitors of store-operated calcium release-activated calcium current (iCRAC). Thus, a possible mechanism by which SphK2 might exert its activating effect on calcium influx could be through reducing the levels of SPH via S1P generation, thereby derepressing the block of iCRAC. This view fits well with a SPH:S1P rheostat mechanism in mast cell responsiveness as proposed by Prieschl and colleages (Prieschl et al., 1999). In these experiments, high concentrations of intracellular SPH prevented leukotriene synthesis and cytokine production and inhibited MAP kinase activation. It should be noted that SPH and lysosphingolipids are also potent inhibitors of PKC activation as well, as shown by the fact that they bind to the regulatory domain preventing interactions of PKC with its stimulatory ligand, diacyglycerol (DAG) (Hannun and Bell, 1989). Thus, upon FcεRI stimulation and activation of SphK2, a decrease in intracellular SPH levels may derepress iCRAC and at the same time cause increased activation of PKCs, like the β isoform, which is essential for mast cell cytokine production and degranulation (Nechushtan et al., 2000; Ozawa et al., 1993).

Among the findings of this study, the demonstrated association between circulating concentrations of S1P and anaphylaxis is most intriguing. These data argue that the concentrations of circulating S1P in mice is primarily determined by SphK1 outside the mast cell compartment and demonstrate that deficiency in this kinase, which results in reduced circulating S1P, is associated with resistance to anaphylaxis even though the mast cells from *Sphk1*$^{-/-}$ mice contain normal intracellular amount of S1P and were unaffected in their responses. Although mast cells can release large amounts of S1P under stimulation, when unchallenged mast cell-deficient mice (W^{-sh}/W^{-sh}) were reconstituted with mast cells, the circulating amounts of S1P were unchanged, demonstrating that cells other than mast cells are likely to be the major contributors to the resting amounts of S1P. The cellular source of circulating S1P has not been clearly determined, but endothelial cells, which secrete SphK1, and platelets or other blood cell types could be likely sources (Venkataraman et al., 2006; Yatomi et al., 1995).

The recent work of Metz et al. (2006) demonstrated that mast cell degranulation elicited by a systemic challenge can be protective in mice. In this study, WT mice and mast cell-deficient mice (W/W^{-v} or W^{-sh}/W^{-sh}) were systemically challenged with snake or bee venom, but only the mast cell-deficient mice were found to succumb to this challenge. The release of the granule protease carboxypeptidase A (CPA) was key to the protective effect, because a neutralizing antibody or silencing (shRNA) of CPA expression caused increased mortality in WT mice. This suggests that mast cell degranulation is likely to be protective rather than detrimental even under conditions of systemic challenge (Rivera, 2006b). This presents a conundrum of why only few allergic individuals, when systemically challenged with an allergen, undergo anaphylactic shock, while most others with similar circulating levels of allergen-specific IgE are resistant (Pumphrey, 2004). The findings of the present study suggest the possibility that the circulating amounts of S1P may be a factor that elicits an overly extensive degranulation of mast cells that is thought to cause the anaphylactic response. Importantly, this work further demonstrates that the in vivo environment in which the mast cell resides is a key determinant of its responsiveness and of susceptibility to anaphylaxis. These findings have important clinical and therapeutic implications in allergic disease, particularly because SphK-specific inhibitors are under development (Kim et al., 2005).

EXPERIMENTAL PROCEDURES

Mice, Liver-Derived Mast Cell Isolation, and Differentiation

Mice were maintained and used in accordance with National Institutes of Health (NIH) guidelines and an animal study proposal approved by the institutional animal care and use committee. The embryos of genetically altered mice (129Sv × C57BL6 mixed background) were obtained at 11.5 days of pregnancy. WT and *Sphk1*$^{-/-}$ embryos littermates were obtained by mating *Sphk1*$^{+/-}$ mice, whereas *Sphk2*$^{-/-}$ and *Sphk1*$^{-/-}$*Sphk2*$^{-/-}$ littermates were obtained by mating *Sphk1*$^{+/-}$/*Sphk2*$^{-/-}$ mice. Genotyping was performed as described (Allende et al., 2004; Mizugishi et al., 2005). Cell progenitors from the embryonic livers were isolated and cultured as described (Kovarova et al., 2006).

Immunoblots and Immunoprecipitations

For immunoprecipitations and immunoblotting procedures, cells (30×10^6 and 10×10^6, respectively) were sensitized with 0.05 μg IgE per million cells for 3 hr in Tyrodes-0.05% fatty acid-free BSA buffer (Olivera et al., 2006), washed, and challenged with 100 ng/ml of Ag (DNP_{36}-BSA). Cells were solubilized as described (Parravicini et al., 2002). Protein concentrations were determined to ensure equal loading. For immunoblots of cell membrane proteins, cells were lysed by freeze-thawing followed by centrifugation at 800 × g for 10 min to remove nuclei and cell debris. The clarified lysates were then centrifuged at 100,000 × g for 1 hr. Recovered membranes were washed with PBS and solubilized in SDS-PAGE sample buffer.

β-Hexosaminidase, Cytokine, LTB_4, and Arachidonic Acid Release Assays

The enzymatic activity of the granule marker, β-hexosaminidase, released to the extracellular media was measured as described (Saitoh et al., 2000) from the supernatants of cells challenged with indicated concentrations of Ag for 10 min.

For cytokine measurements, cells were sensitized overnight (5 to 10×10^6 per sample) with 0.01 μg IgE per million cells and stimulated with the indicated concentration of Ag or alternatively with PMA (40 nM) and calcium ionophore (A23187, 800 nM) in serum-, SCF-, and IL-3-free medium containing 0.05% fatty acid-free BSA and EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics) for 4 hr at 37°C in a CO_2 incubator. For LT measurements, IgE-sensitized cells (2.0×10^6 per sample) were pretreated for 30 min with 10 μg/ml indomethacin and stimulated with the indicated concentrations of Ag in Tyrode's-BSA for an additional 30 min at 37°C. Cells were then centrifuged and the supernatants collected. TNF, IL-6, IL-13, or LTB_4 were measured by specific ELISA. For arachidonic acid release, mast cells (10^6 per sample) were loaded with 0.1 μCi ^{14}C-labeled arachidonic acid and sensitized with IgE overnight at 37°C. Cells were washed with Tyrode's-BSA and activated with 25 ng/ml Ag for the indicated time, and the percent arachidonic acid released in the medium was determined from total cellular content as described (Gomez et al., 2005).

Passive Systemic Anaphylaxis

Mice were sensitized with 3 μg of DNP-specific IgE by retro-orbital injection. 24 hr later, mice were challenged with 300 μg of Ag (DNP_{36}-HSA) or vehicle (PBS) by retro-orbital injection. After 1.5 min, mice were euthanized with CO_2, and blood was immediately withdrawn by cardiac puncture. Plasma histamine concentration was determined with a competitive histamine immunoassay kit (Beckman Coulter).

Nuclear NF-κB DNA-Binding Assay

Cells (9×10^6 cells per sample) were sensitized with 0.05 μg of IgE for 3 hr at 37°C and activated for 20 min with 30 ng/ml Ag or with 40 nM PMA plus 800 nM Ionophore. Nuclear proteins were obtained with a commercial kit (Active Motif), and nuclear NF-κB DNA binding activity was determined as described (Gomez et al., 2005).

Intracellular Calcium Determinations, Calcium Uptake, and IP_3 Measurements

For intracellular calcium measurements, cells were sensitized overnight and loaded with 3 μM FURA-2AM for 20 min, washed, and aliquoted in 96-well plates (9×10^4 cells/well). After 20 min, cells were challenged with Ag (25 to 50 ng/ml), and changes in intracellular calcium were monitored with a microplate fluorescence reader (Opti-Fluor; BMG Labtechnologies). FURA-2 emission at 510 nm during fast excitation between 340 and 380 nm at 37°C was measured, and background fluorescence was determined by adding Mn^{2+}, a quencher of Fura-2 fluorescence, to a final concentration of 5 mM. The ratio of

fluorescence at 340 nm and 380 nm (R) after subtracting the respective background values was calculated for each measurement. The ratio of maximal (R_{max}) and minimal (R_{min}) intracellular concentrations was determined in cells treated with 1% Triton X-100 (max) or 0.5 mM EGTA (min). Intracellular calcium concentrations were calculated from the equation: Free $[Ca^{2+}] = Kd[(R - R_{min}/R_{max} - R)(380\lambda_{min}/380\lambda_{max})]$, where Kd = 224. For calcium uptake, sensitized cells (2×10^6/sample) were stimulated in Tyrodes/BSA for 15 min in the presence of 5 μCi $^{45}Ca^{2+}$. After washing, incorporated $^{45}Ca^{2+}$ was measured by scintillation counting. Intracellular IP_3 was extracted with 20% cold perchloric acid from IgE-sensitized cells (10×10^6/sample) challenged with 25 ng/ml Ag. The acidified samples were centrifuged at 2000 × g for 15 min at 4°C, and the supernatants were neutralized with a 1:1 (v/v) mixture of 1,1, 2-trichlorotrifluoroethane:tri-n-octylamine. The concentration of IP_3 in each sample was measured with a competitive binding assay (IP_3-[^{3}H] Biotrak Assay System, Amersham Biosciences).

RNA Extraction, RT-PCR, and Taqman

Total RNA was isolated from 2 to 4×10^6 cells with a double extraction with Trizol (Invitrogen) according to manufacturer's instructions and further purified with a RNA purification kit (RNeasy, Quiagen). For *Sphk2* alleles, RNA was reverse transcribed with the SuperScript First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen), as specified by the manufacturer, and PCR was performed with primers P1 (5′-ACCACTTATGAGGAGAATCG-3′) and P2 (5′-CACCACGTGGTCCATACAGC-3′). Each PCR cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 2 min of extension at 72°C. For *Sphk1* alleles, purified RNA was reversed transcribed and amplified by PCR in one step (PowerScriptTM One-Step qRT-PCR Mix, Clontech Laboratories, Inc.) with the primers P3 (5′-TGTCACCCATGAACCTGCTGTCCCTGCACA) and P4 (5′-TGAGA AGGCACTGGCTCCTCCAGAGGAACAAG) and exactly according to the thermal cycle conditions specified by the manufacturer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to monitor RNA recovery in each of the reactions.

Sphingosine Kinase Activity and Mass Measurement of S1P and SPH

SphK1 and 2 activities were independently and differentially measured with a 20%–30% overlap exactly as described (Olivera et al., 2006). SPH and S1P recovered from cell extracts or S1P recovered from serum samples were quantified by an enzymatic assay with cell lysates from HEK293 cells overexpressing SphK1 exactly as described (Olivera et al., 2006).

Determination of Mast Cell Numbers

5 ml of RPMI media containing 10% serum was injected in the peritoneum of euthanized mice, and the peritoneal lavage was recovered after the abdomen was lightly massaged. Peritoneal cells were centrifuged and stained in 1% BSA-PBS with antibodies to c-KIT (Allophycocyanin-conjugated [APC]) and a combination of phycoerythrin-conjugated anti-FcεRI plus phycoerythrin-conjugated IgE or with the appropriate IgG isotype control combinations and sorted by flow cytometry with a FACScan (Becton Dickinson). Mast cells (which showed double-positive staining for c-KIT and FcεRI) were quantified as the percent of the total cells in the peritoneal lavage. Samples of dorsal skin and stomach from euthanized mice were fixed in 10% buffered formalin and embedded in paraffin, and sections were stained with toluidine blue at low pH and counterstained with eosine. Metachromatic-stained mast cells were counted in each field (2 mm^2) with a microscope that used 16× objective.

Statistical Analysis

Statistical analysis was performed with PRISM GRAPHPAD Software. For most comparisons, a nonpaired Student's t test was used. For comparing responses of the various mutant cells over time, a two-way Anova test was used, as indicated.

Supplemental Data

Six Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/3/287/DC1/.

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REFERENCES

Allende, M.L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., van Echten-Deckert, G., Hajdu, R., Rosenbach, M., Keohane, C.A., Mandala, S., et al. (2004). Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. J. Biol. Chem. *279*, 52487–52492.

Ammit, A.J., Hastie, A.T., Edsall, L.C., Hoffman, R.K., Amrani, Y., Krymskaya, V.P., Kane, S.A., Peters, S.P., Penn, R.B., Spiegel, S., and Panettieri, R.A., Jr. (2001). Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J. *15*, 1212–1214.

Behn-Krappa, A., and Newton, A.C. (1999). The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation. Curr. Biol. *9*, 728–737.

Birchwood, C.J., Saba, J.D., Dickson, R.C., and Cunningham, K.W. (2001). Calcium influx and signaling in yeast stimulated by intracellular sphingosine 1-phosphate accumulation. J. Biol. Chem. *276*, 11712–11718.

Blom, T., Slotte, J.P., Pitson, S.M., and Tornquist, K. (2005). Enhancement of intracellular sphingosine-1-phosphate production by inositol 1,4,5-trisphosphate-evoked calcium mobilisation in HEK-293 cells: endogenous sphingosine-1-phosphate as a modulator of the calcium response. Cell. Signal. *17*, 827–836.

Choi, O.H., Kim, J.-H., and Kinet, J.-P. (1996). Calcium mobilization via sphingosine kinase in signalling by the FcεRI antigen receptor. Nature *380*, 634–636.

Condrescu, M., Opuni, K., Hantash, B.M., and Reeves, J.P. (2002). Cellular regulation of sodium-calcium exchange. Ann. N Y Acad. Sci. *976*, 214–223.

Edsall, L.C., Van Brocklyn, J.R., Cuvillier, O., Kleuser, B., and Spiegel, S. (1998). N,N-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. Biochemistry *37*, 12892–12898.

Edsall, L., Vann, L., Milstien, S., and Spiegel, S. (2000). Enzymatic method for measurement of sphingosine 1-phosphate. Methods Enzymol. *312*, 9–16.

Galli, S.J., Kalesnikoff, J., Grimbaldeston, M.A., Piliponsky, A.M., Williams, C.M., and Tsai, M. (2005). Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu. Rev. Immunol. *23*, 749–786.

Gomez, G., Gonzalez-Espinosa, C., Odom, S., Baez, G., Cid, M.E., Ryan, J.J., and Rivera, J. (2005). Impaired FcεRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. J. Immunol. *175*, 7602–7610.

Gon, Y., Wood, M.R., Kiosses, W.B., Jo, E., Sanna, M.G., Chun, J., and Rosen, H. (2005). S1P3 receptor-induced reorganization of epithelial

tight junctions compromises lung barrier integrity and is potentiated by TNF. Proc. Natl. Acad. Sci. USA *102*, 9270–9275.

Hannun, Y.A., and Bell, R.M. (1989). Regulation of protein kinase C by sphingosine and lysosphingolipids. Clin. Chim. Acta *185*, 333–345.

Hannun, Y.A., Luberto, C., and Argraves, K.M. (2001). Enzymes of sphingolipid metabolism: from modular to integrative signaling. Biochemistry *40*, 4893–4903.

Itagaki, K., and Hauser, C.J. (2003). Sphingosine 1-phosphate, a diffusible calcium influx factor mediating store-operated calcium entry. J. Biol. Chem. *278*, 27540–27547.

Jolly, P.S., Bektas, M., Olivera, A., Gonzalez-Espinosa, C., Proia, R.L., Rivera, J., Milstien, S., and Spiegel, S. (2004). Transactivation of sphingosine-1-phosphate receptors by FcεRI triggering is required for normal mast cell degranulation and chemotaxis. J. Exp. Med. *199*, 959–970.

Kim, J.W., Kim, Y.W., Inagaki, Y., Hwang, Y.A., Mitsutake, S., Ryu, Y.W., Lee, W.K., Ha, H.J., Park, C.S., and Igarashi, Y. (2005). Synthesis and evaluation of sphingoid analogs as inhibitors of sphingosine kinases. Bioorg. Med. Chem. *13*, 3475–3485.

Kitano, M., Hla, T., Sekiguchi, M., Kawahito, Y., Yoshimura, R., Miyazawa, K., Iwasaki, T., Sano, H., Saba, J.D., and Tam, Y.Y. (2006). Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: regulation of synovial proliferation and inflammatory gene expression. Arthritis Rheum. *54*, 742–753.

Klemm, S., Gutermuth, J., Hultner, L., Sparwasser, T., Behrendt, H., Peschel, C., Mak, T.W., Jakob, T., and Ruland, J. (2006). The Bcl10-Malt1 complex segregates FcεRI-mediated nuclear factor κB activation and cytokine production from mast cell degranulation. J. Exp. Med. *203*, 337–347.

Kovarova, M., Wassif, C.A., Odom, S., Liao, K., Porter, F.D., and Rivera, J. (2006). Cholesterol deficiency in a mouse model of Smith-Lemli-Opitz syndrome reveals increased mast cell responsiveness. J. Exp. Med. *203*, 1161–1171.

Lee, H.S., Park, C.S., Lee, Y.M., Suk, H.Y., Clemons, T.C., and Choi, O.H. (2005a). Antigen-induced Ca^{2+} mobilization in RBL-2H3 cells: role of I(1,4,5)P3 and S1P and necessity of I(1,4,5)P3 production. Cell Calcium *38*, 581–592.

Lee, K.Y., D'Acquisto, F., Hayden, M.S., Shim, J.H., and Ghosh, S. (2005b). PDK1 nucleates T cell receptor-induced signaling complex for NF-κB activation. Science *308*, 114–118.

Liu, H., Sugiura, M., Nava, V.E., Edsall, L.C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000). Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. J. Biol. Chem. *275*, 19513–19520.

Marquardt, D.L., and Walker, L.L. (2000). Dependence of mast cell IgE-mediated cytokine production on nuclear factor-κB activity. J. Allergy Clin. Immunol. *105*, 500–505.

Massberg, S., and von Andrian, U.H. (2006). Fingolimod and sphingosine-1-phosphate—modifiers of lymphocyte migration. N. Engl. J. Med. *355*, 1088–1091.

Mathes, C., Fleig, A., and Penner, R. (1998). Calcium release-activated calcium current (ICRAC) is a direct target for sphingosine. J. Biol. Chem. *273*, 25020–25030.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355–360.

Melendez, A.J., and Khaw, A.K. (2002). Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. J. Biol. Chem. *277*, 17255–17262.

Metz, M., Piliponsky, A.M., Chen, C.C., Lammel, V., Abrink, M., Pejler, G., Tsai, M., and Galli, S.J. (2006). Mast cells can enhance resistance to snake and honeybee venoms. Science *313*, 526–530.

Michell, R.H., Conroy, L.A., Finney, M., French, P.J., Bunce, C.M., Anderson, K., Baxter, M.A., Brown, G., Gordon, J., Jenkinson, E.J., et al. (1992). Inositol lipids and phosphates in the proliferation and differentiation of lymphocytes and myeloid cells. Ciba Found. Symp. *164*, 2–11.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Mol. Cell. Biol. *25*, 11113–11121.

Nechushtan, H., Leitges, M., Cohen, C., Kay, G., and Razin, E. (2000). Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase Cβ. Blood *95*, 1752–1757.

Olivera, A., and Rivera, J. (2005). Sphingolipids and the balancing of immune cell function: lessons from the mast cell. J. Immunol. *174*, 1153–1158.

Olivera, A., and Spiegel, S. (2001). Sphingosine kinase: a mediator of vital cellular functions. Prostaglandins *64*, 123–134.

Olivera, A., Urtz, N., Mizugishi, K., Yamashita, Y., Gilfillan, A.M., Furumoto, Y., Gu, H., Proia, R.L., Baumruker, T., and Rivera, J. (2006). IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses. J. Biol. Chem. *281*, 2515–2525.

Ozawa, K., Szallasi, Z., Kazanietz, M.G., Blumberg, P.M., Mischak, H., Mushinski, J.F., and Beaven, M.A. (1993). Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca2+ and purified isozymes in washed permeabilized cells. J. Biol. Chem. *268*, 1749–1756.

Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L.E., O'Shea, J.J., and Rivera, J. (2002). Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. Nat. Immunol. *3*, 741–748.

Prieschl, E.E., Csonga, R., Novotny, V., Kikuchi, G.E., and Baumruker, T. (1999). The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fcε receptor I triggering. J. Exp. Med. *190*, 1–8.

Pumphrey, R. (2004). Anaphylaxis: can we tell who is at risk of a fatal reaction? Curr. Opin. Allergy Clin. Immunol. *4*, 285–290.

Rivera, J. (2006a). Adaptors discriminate mast-cell cytokine production from eicosanoid production and degranulation. Trends Immunol. *27*, 251–253.

Rivera, J. (2006b). Snake bites and bee stings: the mast cell strikes back. Nat. Med. *12*, 999–1000.

Rivera, J., and Gilfillan, A.M. (2006). Molecular regulation of mast cell activation. J. Allergy Clin. Immunol. *117*, 1214–1225.

Rosen, H., and Goetzl, E.J. (2005). Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat. Rev. Immunol. *5*, 560–570.

Saito, H. (2005). Mast cell-specific genes-new drug targets/pathogenesis. Chem. Immunol. Allergy *87*, 198–212.

Saitoh, S., Arudchandran, R., Manetz, T.S., Zhang, W., Sommers, C.L., Love, P.E., Rivera, J., and Samelson, L.E. (2000). LAT is essential for FcεRI-mediated mast cell activation. Immunity *12*, 525–535.

Saitoh, S., Odom, S., Gomez, G., Sommers, C.L., Young, H.A., Rivera, J., and Samelson, L.E. (2003). The four distal tyrosines are required for LAT-dependent signaling in FcεRI-mediated mast cell activation. J. Exp. Med. *198*, 831–843.

Sanchez, T., and Hla, T. (2004). Structural and functional characteristics of S1P receptors. J. Cell. Biochem. *92*, 913–922.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735–1739.

Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell Biol. *4*, 397–407.

Titievsky, A., Titievskaya, I., Pasternack, M., Kaila, K., and Tornquist, K. (1998). Sphingosine inhibits voltage-operated calcium channels in GH4C1 cells. J. Biol. Chem. *273*, 242–247.

Urtz, N., Olivera, A., Bofill-Cardona, E., Csonga, R., Billich, A., Mechtcheriakova, D., Bornancin, F., Woisetschlager, M., Rivera, J., and Baumruker, T. (2004). Early activation of sphingosine kinase in mast cells and recruitment to FcεRI are mediated by its interaction with Lyn kinase. Mol. Cell. Biol. *24*, 8765–8777.

Venkataraman, K., Thangada, S., Michaud, J., Oo, M.L., Ai, Y., Lee, Y.M., Wu, M., Parikh, N.S., Khan, F., Proia, R.L., and Hla, T. (2006). Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. Biochem. J. *397*, 461–471.

Wang, D., Matsumoto, R., You, Y., Che, T., Lin, X.Y., Gaffen, S.L., and Lin, X. (2004). CD3/CD28 costimulation-induced NF-κB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IκB kinase β to the immunological synapse through CARMA1. Mol. Cell. Biol. *24*, 164–171.

Welle, M. (1997). Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. J. Leukoc. Biol. *61*, 233–245.

Yatomi, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1995). Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood *86*, 193–202.

Zemann, B., Kinzel, B., Muller, M., Reuschel, R., Mechtcheriakova, D., Urtz, N., Bornancin, F., Baumruker, T., and Billich, A. (2006). Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. Blood *107*, 1454–1458.